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ORIGINAL ARTICLE

Isolation of four rice seed-specific promoters and evaluation of endosperm activity

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Abstract Cereal grains are major targets for genetically improving the nutritional value of food and for producing recombinant proteins. Strong and tissue-specific promoters are highly desired for effectively controlling expression in the seed or endosperm. In this study, we isolated four rice promoters from the 5′ upstream region of putative seed-specifically expressed genes: *PROLAM26, RAL2, RAL4* and *CAPIP*. By generating transgenic rice plants carrying promoter-reporter constructs, we found these four promoters to be specifically expressed in seeds, with three having endosperm-specific or -preferential activity. The strength of each promoter in the endosperm was determined and compared to a constitutively expressed *OsACTIN* promoter and an endosperm-specifically expressed *Glu4-B* promoter in single-copy transgenic plants. The promoter of *RAL2* exhibited relatively high activity, and the promoters of *RAL4* and *CAPIP* exhibited activities comparable with those of *OsACTIN* and *Glu4-B*. In addition, monitoring activities in high-generation (T_3–T_4) homozygous progeny of single-copy plants revealed maintenance of expression for all four promoters, with no evidence of silencing. Taken together, our findings offer four stable rice seed-specific promoters of different strengths for endosperm expression.

Keywords Endosperm specific · Promoter · Rice · Transgene · Biotechnology

Introduction

Rice is the most preferred staple food in both East and South-east Asia, providing nutrition to more than half of the human population (Khush 2005). Although rice grains are a main source of calories and protein for humans, the protein content is significantly lower than that of other cereals. Furthermore, rice grains contain low levels of amino acids (e.g., lysine and threonine) and essential vitamins and micronutrients (Zimmermann and Hurrell 2002). The endosperm is the main storage organ for starch and protein in cereal crops, and qualitative and quantitative enhancement of the rice endosperm is an important breeding objective. In recent years, genetic engineering has achieved some notable advances in the field of rice breeding, improving the protein and micronutrient contents of rice endosperm *via* expression of exogenous biosynthesis genes or transporters (Boonyaves et al. 2016; Lee et al. 2005; Ye et al. 2000). Moreover, the rice endosperm is considered to be an ideal expression platform for the production of recombinant proteins (Ou et al. 2014; Takaiwa et al. 2007). Compared to other expression systems, rice endosperm bioreactors have several advantages, such as a low cost, clear genetic background, large expression capacity, ease of scale-up production and high level of biosafety (Greenham and Altosaar 2013; Sack et al. 2015). Unlike other tissues, the endosperm naturally functions as a storage organ, providing

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sub-cellular storage compartments for foreign recombinant proteins. In addition, the endosperm shows clear superiority over other organs for applications that involve accumulating, preserving, purifying and delivering recombinant proteins, especially bioactive drugs and vaccines. Indeed, an increasing number of economically important recombinant proteins are being produced in rice endosperm systems via a biotechnology approach (Bundó et al. 2014; Kudo et al. 2013; Patti et al. 2012; Vamvaka et al. 2016).

Gene promoters directly determine the temporal and spatial distribution and levels of the corresponding transcripts, thus providing an efficient and economic tool for manipulating the expression pattern of target genes in plant biotechnology research. Some strong heterologous constitutive promoters, such as the cauliflower mosaic virus (CaMV) 35S promoter, nopaline synthase (NOS) promoter and maize ubiquitin promoter (P_{ZmUbi}), are widely used in rice genetic modification (Battraw and Hall 1990; Cornejo et al. 1993). However, heterologous promoters may have sequence structures that differ from that of the rice genome and could thus possibly be deactivated (Kilby et al. 1992; Linn et al. 1990). To avoid this risk, several highly active promoters, such as the *OsACTIN1* promoter (P_{ACT}), *OsCc1* promoter and *OsDHAR1* promoter (P_{OsCon1}), have been isolated and applied in rice to drive constitutively high expression of transgenes (Gao et al. 2014; Jang et al. 2002; McElroy et al. 1990). Although constitutive promoters are typically strong, their activity is often not satisfactory for seed expression requirements (Drakakaki et al. 2000). In addition, due to potential interference of plant growth and development through non-targeted expression of foreign genes and the public demand of less intrusive transgene expression, strong endosperm-specific promoters could be better candidates for biotechnology applications in rice grains (Bucchini and Goldman 2002; Desai et al. 2010).

The endosperm is the main component of the seeds of monocot plants, and plant endosperm-specific promoters are typically obtained from cereal storage protein genes. Some endosperm-specific or -preferential promoters from rice have been isolated and characterized, most of which are glutelin promoters (Kawakatsu et al. 2008; Qu and Takaiwa 2004; Qu et al. 2008; Urriola and Rathore 2014). These sequences confer high expression levels in the endosperm of rice and other cereals and are therefore frequently used in molecular breeding and bioreactors. For example, the rice glutelin *Glu4-B* promoter ($P_{Glu4\text{-}B}$) was chosen to control specific and strong endosperm expression of foreign genes, such as phytoferritin genes, folate (Vitamin B9)-binding proteins, antihypertensive peptide and hepatitis B virus (HBV) surface antigen (Blancquaert et al. 2015; Mejima et al. 2015; Oliva et al. 2013; Qian et al. 2007; Wakasa et al. 2011). Although some endosperm promoters have been functionally identified, there is still a paucity of tissue-specific promoters for strong expression. Here, we report the isolation of four novel endosperm-specific/-preferential promoters from the rice genome. Their activities were quantitatively analyzed in transgenic rice plants using the reporter gene *β-glucuronidase* (*GUS*). Our results suggest that these promoters could be regarded as potential alternative elements for the transgenic engineering of rice and other cereals.

Materials and methods

Plant material

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) plants were used for promoter isolation and for *Agrobacterium*-meditated transformation. Rice seeds were germinated on solid 1/2 Murashige and Skoog (MS) medium and incubated for 10 days in a growth chamber under a light/dark cycle of 16 h/8 h at 28 °C. The seedlings were then transferred to soil and grown in a greenhouse at 25–30 °C. Mature seeds were collected after 105 days.

RNA extraction and RT-PCR

Total RNA was extracted from the leaves, roots and stems of plants at 60 days after germination (DAG), flowers, mature seed endosperm, and vegetative tissues of 10-DAG seedlings, 60-DAG plants, and 90-DAG plants using an RNAprep Pure Plant Kit (Tiangen, China). RNase-free DNase I was used to eliminate any genomic DNA, and cDNA was synthesized from approximately 2 μg RNA using Fast Quant RT Kit (Tiangen, China). Semi-quantitative PCR analysis was performed with gene-specific primers and Easy Taq PCR SuperMix (Transgen, China). A housekeeping gene, *OsACTIN1*, was employed as an internal control. Amplification was carried out through initial denaturation at 95 °C for 2 min, followed by 23 cycles (for the internal control) or 28 cycles (for gene detection) of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s. The amplification products from each PCR reaction were separated on a 2.5 % (w/v) low-melting agarose gels.

Promoters cloning and vectors construction

According to the genomic sequence of the rice *PROLAM26, RAL2, RAL4* and *CAPIP* genes, approximately 1.7–2.0 kb immediately upstream of the translational initiation site (ATG) was designated as the promoter (P_{Pro26}, P_{RAL2}, P_{RAL4} and P_{CAPIP}), respectively. To isolate these promoters, genomic DNA was extracted from the leaves of 10-DAG rice seedlings using a DNAsecure Plant Kit (Tiangen, China). PCR was carried out through initial denaturation at

95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s using High-fidelity Fly DNA Polymerase (Transgen, China). After electrophoresis, fragments of the desired size were cloned into a pEASY-T vector (Transgen, China) and sequenced. The sequence-confirmed promoters were digested with the restriction enzymes listed in Supplemental Table 1 and fused upstream of the *GUS* gene in the pCAMBIA1391 vector at the corresponding sites. A 2181-bp and a 1474-bp sequence of P_{ACT} and $P_{Glu\text{-}4B}$ were cloned (McElroy et al. 1990; Patti et al. 2012). All primers used for promoter cloning and plasmid construction are listed in Supplemental Table 1.

Rice transformation

Constructs were introduced into *Agrobacterium-tumefaciens* strain EHA105, and embryonic calli of rice were transformed following a previously reported protocol (Duan et al. 2012). Transgenic plants were regenerated using 50 mg/L hygromycin selection. T_0 plants carrying a single-copy T-DNA insertion were screened using a Taqman Real-time PCR assay following a previously described method (Yang et al. 2005). For each construct, two to three independent plants with the representative expression pattern were strictly self-pollinated, and homozygous T_3 and T_4 plants were used for the ensuing analysis.

Histochemical GUS staining and quantitative GUS assay

For histochemical analysis, plant tissues were vacuum infiltrated for 15 min and then incubated at 37 °C in X-gluc buffer containing 50 mM sodium phosphate, pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 10 mM Na_2-EDTA, 0.1 % (v/v) Triton X-100 and 1 mM X-gluc. The incubation time varied from 30 min to overnight depending on the abundance of GUS activity. After staining, the samples were repeatedly incubated with 70 % ethanol for chlorophyll removal.

Fluorometric assays of GUS activity were performed following a standard protocol. Approximately 100 mg endosperm cells were separated from the mature seed and ground in liquid nitrogen. Total proteins were extracted in extraction buffer (100 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.2 % β-mercaptoethanol, 25 μg/mL PMSF and 25 μg/mL protease inhibitor cocktail). The homogenate was centrifuged at 12,000 rpm at 4 °C for 10 min, and the supernatant was collected. A 20-μL aliquot of the supernatant was mixed with 180 μL assay buffer containing 4-methylumbelliferyl β-D-glucuronide (MUG) substrate and incubated at 37 °C. The reactions were stopped by the addition of Na_2CO_3, and fluorescence signals were detected using a Fluoroskan Ascent FL (Thermo-Fisher, USA) at an emission wavelength of 455 nm and an excitation of 365 nm. GUS enzyme activity is expressed as pmol of 4-methylumbelliferone (MU) produced per minute per microgram of protein.

Results

Four candidate genes showing tissue-specific expression

To identify strong endosperm-specific genes, we first applied the Genevestigator anatomy analysis tool to screen rice transcriptome collections (Hruz et al. 2008). Four candidates, including a prolamin precursor gene *PROLAM26*(*LOC_Os07g10580*), seed allergenic protein RA5/RA14/RA17 precursor genes *RAL2* (*LOC_Os07g11330*) and *RAL4* (*LOC_Os07g11380*) and a gene of unknown function, *CAPIP*(*LOC_Os06g33640*), were selected. To confirm the spatial expression pattern of these genes, transcript abundance in different tissues was examined by semi-quantitative RT-PCR. As shown in Fig. 1, mRNA for *PROLAM26, RAL2* and *RAL4* was only detected in the endosperm of mature seeds and not in mature leaves, roots, or stems. Furthermore, the transcripts were examined in vegetative tissues at different developmental stages, none of which could be detected in 10 DAG seedlings, 60-DAG plants or 90-DAG plants (Supplemental Fig. 1). Using primers for *CAPIP*, we also found strong signals in the endosperm, with weaker expression in other tissues. As the *CAPIP* gene exhibits extremely high similarity to other sequences (*OsPYL8, LOC_Os06g33690*) in the rice genome, it is technically difficult to design primers to distinguish among them. Therefore, the RT-PCR result represents the expression of the two

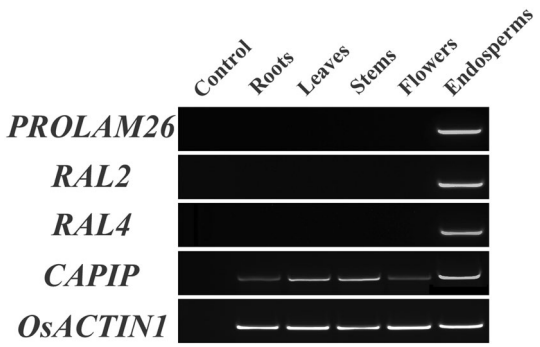

Fig. 1 Expression profile of the rice PROLAM26, RAL2, RAL4 and CAPIP genes by semi-quantitative RT-PCR analysis. Total RNA was isolated from the roots, leaves, stems of 60-DAG plants, flowers and endosperm and reverse transcribed into cDNA for PCR. A 23-cycle PCR amplification using specific primers for the housekeeping gene *OsACTIN* was used to normalize the samples. The spatial patterns of the transcripts of *PROLAM26, RAL2, RAL4* and *CAPIP* were detected using a 28-cycle amplification

transcripts together, suggesting that at least one gene is predominantly expressed in the endosperm.

Promoter isolation and sequence analysis

To investigate whether the promoters of the selected genes could be used to control endosperm expression in rice, regulatory regions of 2062, 1768, 1942 and 1750 bp immediately upstream of the translation start site (ATG) (including the 5′ untranslated region (UTR)) of the *PROLAM26, RAL2, RAL4* and *CAPIP* genes, respectively, were amplified from rice genomic DNA (see the Material and Methods section). The amplified promoter fragments were validated with Nipponbare genome sequences, and no variation was found within the isolated regions. The promoters were separately cloned upstream of a *GUS* reporter gene on the binary vector pCAMBIA 1391 to generate a promoter::*GUS* fusion construct. As positive controls, a widely used strong constitutive promoter from rice, P_{ACT} (McElroy et al. 1990), and a previously identified endosperm-specific expressed promoter, $P_{Glu\text{-}4B}$ (Qu and Takaiwa 2004), were cloned into the binary vector.

Various cis-elements related to endosperm/seed-specific expression have been identified, and using the PLACE scan tool, we found several related cis-elements in the sequences of the isolated promoters (Higo et al. 1999). As shown in Fig. 2-box elements, which are involved in tissue specificity for a dicot seed storage protein promoter (Stålberg et al. 1996), are found in all four promoters. In addition, three, one, one and four copies of the core element for endosperm expression, the −300 core sequence (Colot et al. 1987), are present in P_{Pro26}, P_{RAL2}, P_{RAL4} and P_{CAPIP}, respectively, and, three and two copies of another element related to specificity for a monocot seed storage protein, the Prolamine box (P-box) (Wu et al. 2000), are contained within P_{Pro26} and P_{CAPIP}, respectively. All related tissue-specific elements are found in $P_{Glu\text{-}4B}$. This abundance of seed-specific cis-elements suggests that these promoters may have tissue specificity.

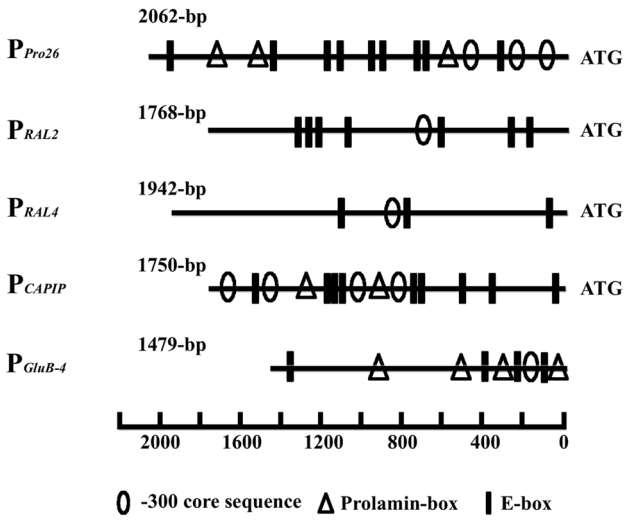

Fig. 2 Schematic representation of the location of cis-elements in the promoters. *Numbers* indicate the positions of nucleotides relative to the translation start site (ATG). The *triangle* represents the prolamin box, the r*ectangle* represents the −300 core sequence, and the *circle* represents the E-Box

Analysis of the promoter activity in a transgenic rice T_0 generation

To assess the activity of the selected promoters, promoter::*GUS* constructs were separately introduced into *japonica* rice (Nipponbare) via *Agrobacterium*-mediated transformation. More than 20 independent plants were generated for each construct, and transformation was confirmed using PCR. To determine the tissue specificity of the promoters, GUS activity in the leaves, stems, roots, flowers and mature seeds of in all transgenic plants was detected by histochemical staining (Table 1). GUS staining was robustly restricted to seed tissue (proper expression) in 48.0, 76.9, 81.8 and 62.5 % of the P_{Pro26}, P_{RAL2}, P_{RAL4} and P_{CAPIP} T_0 populations, respectively. In the remaining plants, GUS activity was either not detected or only detected in tissues other than seeds (improper or null expression). Moreover, the spatial pattern of seed expression for the four promoters differed, though they were all detected in the endosperm. In P_{RAL4} transgenic plants, staining was restricted to endosperm cells. In contrast, blue color was more intense in the embryo and aleurone compared to the endosperm in P_{Pro26} transgenic plants, and pale blue staining was observed in the margin of the embryo of P_{RAL2} and P_{CAPIP} plants (Fig. 3a). To further evaluate the activities of the promoters, single-copy (Supplemental Table 2) transgenic plants with proper expression patterns were screened, and GUS activity was quantitatively determined in the endosperm of the seeds from

Table 1 Tissue specificity of promoters in the T_0 generation

GUS expression[a]	Transgenic plants of the promoter::*GUS* construct[b]			
	P_{PRO26}	P_{RAL2}	P_{RAL4}	P_{CAPIP}
Proper	12	20	18	15
Improper	10	1	4	6
Null	3	5	0	3

[a]GUS expression patterns were determined by overnight X-Gluc incubation. Proper refers to GUS staining restricted to seeds; improper refers to GUS staining in a non-target tissue, such as leaves, stems, roots and flowers; null refers to no staining in the tested tissues

[b]Number of T_0 independent plants with the corresponding expression pattern

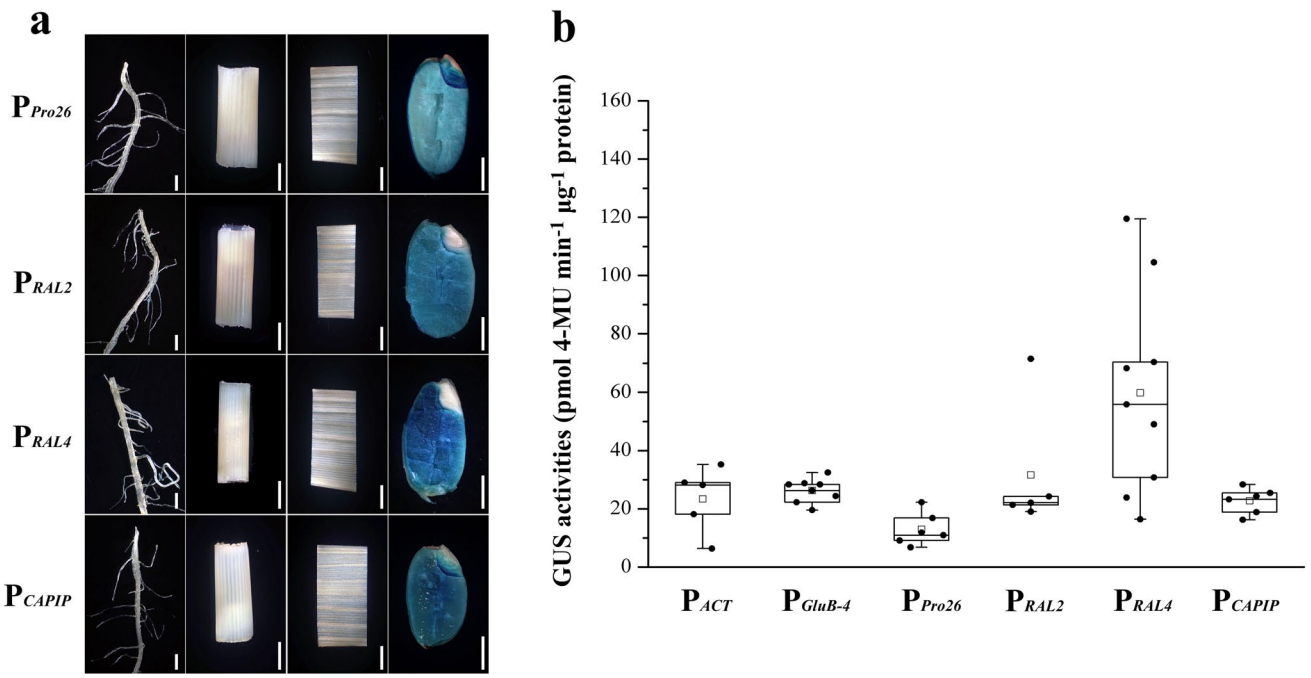

Fig. 3 Expression patterns of PPRO26, PRAL2, PRAL4 and PCAPIP in transgenic plants. **a** Histochemical staining of GUS activity driven by P_{PRO26}, P_{RAL2}, P_{RAL4} and P_{CAPIP} in different tissues. In transgenic plants with proper expression, no obvious GUS staining was detected in the roots, stems and leaves (from *left* to *right*) of 60-DAG plants after 24 h of X-gluc incubation, but strong blue staining could be observed in longitudinal sections of mature seeds only after 30 min to 1 h of incubation. *Scale bars* represent 2 mm. **b** GUS activity for various promoters in the endosperm of mature seeds of single-copy transgenic plants. GUS activity is expressed as pmol 4-MU per min per microgram protein. The *dot* indicates the GUS activity of promoters in each plant. The *whisker cap* represents 10th/90th percentiles. The median is depicted by the line, and the box represents outliers of 10th/90th percentiles

these plants (Fig. 3b). Based on the expression strength, the promoters could be divided into two groups. P_{RAL4} showed high activity, with a mean GUS activity of 59.8 ± 35.2 pmol 4-μ min^{-1} μg^{-1} protein in the nine individual plants of the P_{RAL4} transformants. The other promoters, namely, P_{Pro26}, P_{RAL2} and P_{CAPIP}, showed relatively lower expression: the average GUS activities were 12.9 ± 5.7, 29.6 ± 22.4 and 22.7 ± 4.5 pmol 4-MU min^{-1} μg^{-1} protein in six, five and six independent plants carrying the corresponding construct. Expression levels of the control promoters P_{ACT} and P_{Glu-4B} were also determined in five and eight plants, respectively, showing average activities of 23.4 ± 11.3 and 26.3 ± 4.1pmol 4-MU min^{-1} μg^{-1} protein, slightly lower than that of the P_{RAL4} plants but comparable to the expression levels of P_{RAL2} and P_{CAPIP} transgenic plants.

Analysis of the promoter activity in high-generation transgenic rice

To analyze promoter expression patterns in later generations, two to three single-copy plants of the transgenic plants of each construct with proper and representative expression patterns were strictly self-pollinated to generated homozygous plants. Promoter expression was monitored in late generations (T_3 and T_4). According to histochemical staining, GUS activity remained specific to seeds and was not detected in vegetative organs (data not shown). To further evaluate the strength of the promoters, GUS activity in the endosperm of the high-generation seeds was quantitatively determined. As indicated in Fig. 4, the promoter activity of the same construct in the T_3 and T_4 generations was not significantly different ($P < 0.05$, *t test*), suggesting

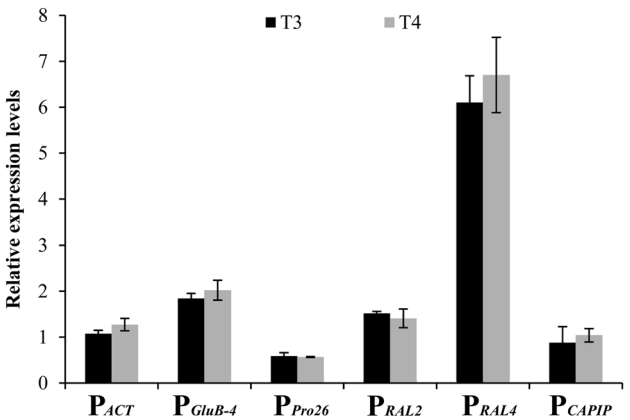

Fig. 4 Quantitative analysis of promoter activity in high-generation transgenic plants. GUS activity was monitored in the endosperm of mature seeds of T_3 and T_4 homozygous single-copy plants. Data *bars* represent the mean ± SD of biological replications

stable expression in these later generations of the transgenic plants. P_{RAL4} showed strong activity, exhibiting 4.1- and 3.7-fold greater GUS expression than in P_{ACT} in the T_3 and T_4 generations, respectively. Similar to the early generation, the expression levels of P_{RAL2} and P_{CAPIP} were comparable to those of P_{ACT} and P_{GluB-4} in the T_3 and T_4 plants. However, the strength of P_{Pro26} was much lower, with 58.7 and 44.7% of the GUS activity of P_{ACT} in the T_3 and T_4 generations, respectively.

Discussion

Seed-specific promoters have broad applications, such as tissue-specific targeting of industrial and pharmaceutical compounds, better functional quality of milled grain, and development of transgenic seeds with improved nutritional quality. Several endosperm-specific or -preferential promoters have been isolated from rice, maize, barley and other crops (Chen et al. 2007; Gunadi et al. 2016; Kawakatsu et al. 2008). The promoters of *Glutelin* genes from rice, *Glutenin* genes from wheat and *Hordein* genes from barley have been exploited for producing recombinant proteins in grains (Furtado et al. 2008; Kawakatsu and Takaiwa 2010; Lamacchia et al. 2001; Stöger et al. 2001). Nonetheless, additional promoters are still needed because the various purposes of transgene expression require a range of activities and temporal patterns. In addition, a battery of different promoters are required to stack multiple transgenes to avoid homology-dependent gene silencing caused by the repetitive use of the same promoter (Potenza et al. 2004). In this study, we isolated four novel rice promoters from putative seed-specific genes. By examining promoter-reporter transgenic plants, activity of all four promoters was found exclusively in seeds and not in other vegetative tissues, such as roots, leaves, sheaths and stems. Although these promoters are specifically or primarily expressed in the endosperm, the details of their spatial patterns differ. Prolamin is a major component of the total seed storage proteins of monocots (Shewry and Halford 2002), and many studies have employed the prolamin promoter to efficiently produce recombinant proteins in cereal grains (Iizuka et al. 2014; Ogo et al. 2014; Wakasa et al. 2015; Wakasa and Takaiwa 2013). In rice, 10, 13 and 16 kDa prolamin gene promoters exhibit endosperm-preferential expression, with strong activities in the aleurone and outer portion of the endosperm but weaker activities in the inner portion of the endosperm (Qu and Takaiwa 2004). P_{Pro26} is also a seed-specific promoter, though it does show high expression in the embryo, with weaker, evenly distributed activity in the endosperm. P_{RAL4} is specifically expressed in the endosperm, with the strongest activity among the four promoters studied here. Indeed, the activity of P_{RAL4} was even several fold higher than that of the strong promoters P_{Glu-4B} and P_{ACT}, suggesting that it may serve as an ideal alternative in genetic engineering. P_{RAL2} and P_{CAPIP} expression was also high in the endosperm, though slight activity could be detected along the edge of the embryo, suggesting that these are endosperm-preferential but not -specific promoters. Although P_{RAL2} and P_{CAPIP} exhibited relatively lower activity than P_{RAL4}, their activity was nonetheless still comparable to that of P_{ACT}. Most monocot seed-specific promoters have been isolated from genes encoding seed storage proteins. A previous genome-wide analysis indicated that some rice *RALs* should be restrictively expressed in the endosperm (Nie et al. 2013). Consistently, our results showed two rice *RAL* promoters are specifically or preferentially expressed in the endosperm with strong activity, thus providing a potential direction for screening new endosperm-specific promoters. Moreover, it has been reported that *OsPYL8* and *CAPIP* (also known as *OsPYL7*) transcripts mainly accumulate in the embryo, partly in the endosperm, and slightly in vegetative tissues (Tian et al. 2015). As the activity of P_{CAPIP} suggested that the *CAPIP* gene may be preferentially expressed in the endosperm, *OsPYL8* transcripts would accumulate in the embryo and also in vegetative tissues. The endosperm is not a tissue generally targeted by ABA; thus, we speculate that *OsPYL8* may be involved in ABA sensing and signaling, more so than *OsPYL7*, which is consistent with the fact that *OsPYL8* exhibits stronger interactions with rice PP2Cs (Tian et al. 2015).

It is a common strategy to elucidate the activity of a promoter by generating stably transformed plants. However, insertions often are multiple copies that are randomly dispersed throughout the genome by *Agrobacterium*- or biolistic-mediated transformation. Higher copy numbers may lead to transgene instability and gene silencing (Hernandez-Garcia and Finer 2014; Tang et al. 2007), whereas random insertions may result in great variability in expression patterns due to positional effects and potential loss of chromatin-mediated regulation. In addition, promoter expression may vary between different generations of the same transformation event (Chen et al. 2013). Therefore, to evaluate potential utility in plant biotechnical applications, promoter activities need to be progressively examined in several generations of single-copy events. Although many engineering-purpose promoters have been reported, very few, including seven rice constitutive promoters and the stress-inducible *OsNCED3* promoter, have been examined with regard to long-term expression stability in single-copy populations (Bang et al. 2012, 2015; Park et al. 2012). In this study, we observed variation in promoter expression in the T_0 population and further monitored the activity of single-copy events of early (T_0) and late (T_3 and T_4) generations. Our results indicated that all four promoters are seed specific and have strong activity in the endosperm in single-copy populations,

with stable expression between generations. Therefore, these newly isolated promoters have potential value in the future for stable grain-specific gene expression in efforts to enhance nutritional value and/or for bioreactor production.

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Author contributions R.X., D.L. and J.L. cloned promoters and constructed vectors. H.L., Y.Y., R.Q. and L.L. performed rice transformations. R.X., D.L. and R.Q. determined the gene expression level and GUS biochemical activities. R.X. and P.W. analyzed the data and drew illustrations. P.W. and J.Y. designed experiments and wrote the manuscript.

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